The Autoantigen of Anti-p200 Pemphigoid Is an Acidic Noncollagenous N-Linked Glycoprotein of the Cutaneous Basement Membrane

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Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease characterized by autoantibodies to a 200-kDa protein (p200) of the dermal–epidermal junction (DEJ). p200 has been demonstrated to be distinct from all major DEJ autoantigens and is thought to be important for cell-matrix adhesion. This study provides the first biochemical characterization of p200. Differential extraction experiments demonstrated that efficient recovery of p200 from the dermis was strongly dependent on the presence of reducing agents, suggesting that it forms highly insoluble oligomers and/or is extensively cross-linked to other extracellular matrix components by disulfide bonding. p200 was resistant to digestion with bacterial collagenase, whereas this treatment did degrade major collagenous proteins of the dermis, including type I, VI, and VII collagen. This finding firmly established the noncollagenous nature of p200. *N*-Glycosidase F reduced the molecular size of the p200 autoantigen from 200 to 190 kDa without decreasing its immunoreactivity. In contrast, digestion of p200 with neuraminidase, *O*-glycosidase, chondroitinase ABC, and heparitinase I had no effect on its electrophoretic mobility. These data suggest that the p200 molecule contains N-glycans but lacks O-linked oligosaccharides and chondroitin/heparan sulfate side chains. Two-dimensional gel electrophoresis demonstrated that p200 is an acidic protein with an isoelectric point of 5.4 to 5.6. Six different p200-specific sera recognized an identical protein spot of two-dimensionally separated dermal extracts, confirming that patients with this novel autoimmune disease indeed form a single pathobiochemical entity. ***Key words: autoantibody/collagen/dermal–epidermal junction/glycosylation/two-dimensional gel electrophoresis. J Invest Dermatol 121:1402–1408, 2003***

Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease first described in 1996 (Zillikens *et al*, 1996; Chen *et al*, 1996). Immunopathologically, it is characterized by linear deposits of IgG and C3 along the dermal–epidermal junction (DEJ) as detected by direct immunofluorescence microscopy of perilesional skin biopsies. Indirect immunofluorescence of patients' sera demonstrates circulating IgG autoantibodies labeling the dermal side of NaCl-split normal skin. The antigenic target of these antibodies is a 200-kDa protein (p200) of the human dermis that is thought to be important for cell-matrix adhesion (Zillikens *et al*, 1996; Kawahara *et al*, 2000). Ultrastructural studies localized p200 to the lower portion of the lamina lucida of the cutaneous basement membrane (Zillikens *et al*, 1996; Chen *et al*, 1996; Egan *et al*, 2002). While p200 was demonstrated to be immunologically distinct from all major autoantigens of the DEJ, including bullous pemphigoid (BP) antigens 180 and 230, α6β4 integrin, laminin 5 and 6, and type VII collagen (Zillikens *et al*, 1996, 2000; Kawahara *et al*, 2000), little is known about its structure and biochemical properties. In this study, we investigate the association of p200 with the extracellular matrix (ECM) and the presence of collagenous domains and carbohydrate moieties within this molecule. In addition, we demonstrate that IgG4 autoantibodies from six patients with anti-p200 pemphigoid recognize the same unique protein of two-dimensionally separated dermal extracts.

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Abbreviations: BP, bullous pemphigoid; DEJ, dermal–epidermal junction; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Patient and rabbit sera and monoclonal antibodies In this study, we used sera from six patients with anti-p200 pemphigoid. Four of these patients have been described in detail elsewhere (Zillikens *et al*, 1996, 2000; Mascaro *et al*, 2000; Egan *et al*, 2002). All patients fulfilled the following criteria: (1) linear deposits of IgG and C3 along the DEJ as detected by direct immunofluorescence of perilesional skin biopsies; (2) linear deposition of IgG along the dermal side of NaCl-split normal human skin by indirect immunofluorescence microscopy; and (3) circulating antibodies against a 200-kDa protein by immunoblotting of extracts of normal human dermis. Rabbit antibodies to human type I and type VI collagen were purchased from Rockland (Gilbertsville, PA). Rabbit serum to human proteoglycan I (biglycan) was provided by H. Kresse (Münster, Germany). Rabbit serum SA8010 was raised against recombinant BP180 NC16A (Sitaru *et al*, 2002). Monoclonal antibodies 1A8c and BM515 were

raised against BP180 and the α3 chain of laminin 5, respectively (Hirako *et al*, 1996, 2003). Monoclonal antibody LH7.2 against the NC1 domain of type VII collagen was purchased from Sigma (St. Louis, MO) and monoclonal antibody A1 against the γ1 chain of laminin 1 was from Neomarkers (Fremont, CA). For the experiments conducted, we obtained institutional approval (Institutional Board Project # 37/98) issued by the director of the ethics committee at the Medical Faculty of the University of Wuerzburg. We obtained informed consent from all patients whose material was used in the study, in adherence to the Helsinki Principles.

Immunoblotting and determination of subclass distribution of p200-specific autoantibodies Immunoblotting of dermal extracts and other substrates was performed as described previously (Zillikens *et al*, 1996). Unless otherwise specified, all immunoblotting studies with serum samples from patients with anti-p200 pemphigoid were performed using anti-IgG4 secondary antibodies (see below). Subclass distribution of p200-specific autoantibodies was determined using peroxidase-conjugated mouse anti-human IgG1 (clone 8c/6-39), antihuman IgG2 (clone HP6014), anti-human IgG3 (clone HP6050), and anti-human IgG4 (clone HP6023) (all from The Binding Site, Birmingham, UK) following a previously published protocol (Chimanovitch *et al*, 1999). Sensitivities of these secondary monoclonal antibodies, used at their working dilutions, had been shown to be comparable by immunoblot analysis with equal amounts of human myeloma proteins (Chimanovitch *et al*, 1999).

Preparation of dermal extracts, differential extraction, and solubility studies Fresh normal human skin was obtained from plastic surgery and incubated in phosphate-buffered saline, pH 7.2, supplemented with 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM ethylenediaminetetraacetic acid (EDTA) at 4°C for 96 h. After dermal–epidermal separation, the epidermis was peeled off and discarded. The epidermal surface of the dermis was extensively washed with phosphate-buffered saline and extracted with buffer A (12.5 mM Tris-HCl, pH 7.0, 8 M urea, 6% β-mercaptoethanol, 1 mM PMSF, and 5 mM EDTA) at room temperature. After 1 h, the dermal extract was collected and centrifuged, and the supernatant was stored at −80°C. For differential extraction experiments, deepidermized dermis was prepared as described above and then extracted with buffer B (same as buffer A but without β-mercaptoethanol) followed by buffer A. For some experiments, the epidermal surface of the dermis was digested with bacterial collagenase as described below and subsequently extracted with buffers B and A. To determine the solubility of extracted protein, dermal extracts were alkylated by addition of 100 mM iodoacetamide for 1 h in the dark and subsequently dialyzed against 4, 2, or 1 M urea (all solutions buffered with 50 mM Tris-HCl, pH 7.5), RIPA buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), or Tris-buffered saline (same as RIPA but without detergents), respectively. Dialyzed extracts were centrifuged at $100{,}000 \times g$ for 2 h, and both supernatants and pellets were analyzed for reactivity with anti-p200 sera by immunoblotting.

Cell culture Normal keratinocytes and fibroblasts were cultured from outgrowing cells of human neonatal foreskins as described previously (Zillikens *et al*, 1996). HaCaT keratinocytes were provided by N.E. Fusenig (Heidelberg, Germany) and grown as described by Boukamp *et al* (1988). Confluent monolayers of keratinocytes or fibroblasts were extracted with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 6% β-mercaptoethanol) supplemented with 5 mM EDTA and 1 mM PMSF. Extracts were pulse-sonicated on ice and centrifuged, and the supernatant was collected. Proteins in the medium conditioned by HaCaT keratinocytes or fibroblasts were concentrated by ammonium sulfate precipitation as described (Kromminga *et al*, 2000). Cryosections of organotypic co-cultures of human keratinocytes and fibroblasts harvested at 3 wk (Smola *et al*, 1998) were provided by N.E. Fusenig.

Proteolytic digests Collagenase digestion was performed as described by Giudice *et al* (1992) with some modification. Dermal extracts were dialyzed against collagenase buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM $CaCl_2$). Protease inhibitors (1 mM PMSF, 1 mM AEBSF, 10 μg/mL leupeptin, 10 μg/mL antipain, 15 μg/mL pepstatin A, 15 μg/mL chymostatin) and 1 Mandl U/mL of collagenase from *Clostridium histolyticum* (Amano Pharmaceuticals, Japan) were added to the dialysate, and after incubation at 37°C for various periods of time, the reaction was stopped by addition of 5 × Laemmli sample buffer, containing 100 mM EDTA, and boiling for 5 min. Negative controls included dermal extracts incubated at 37°C without collagenase and collagenase digestion of bovine serum albumin (Sigma). For tissue digestion, collagenase buffer containing protease inhibitors and 100 Mandl U per mL collagenase was applied to the epidermal surface of deepidermized dermis and incubated at 37°C 4 h. For digestion with bacterial protease V8 (Glu-C), dermal extracts were dialyzed against 0.01% SDS in 25 mM NH_4HCO_3 (pH 7.8) and protease V8 from *Staphylococcus aureus* (Roche, Mannheim, Germany) was added at an enzyme to substrate ratio of 1:100. After incubation at room temperature for various periods of time, the reaction was stopped by addition of 5 × Laemmli sample buffer and boiling. The products of V8 digestion were separated in 4% to 20% gradient gels (Bio-Rad, Hercules, CA) and studied by immunoblotting.

Deglycosylation studies To investigate the presence of N-linked carbohydrate moieties within the p200 molecule, dermal extracts were dialyzed against 20 mM Na_3PO_4, pH 7.2, supplemented with 0.2% SDS, 1% β-mercaptoethanol, 15 mM EDTA and then denatured at 100°C for 5 min. After addition of 1% Nonidet P-40 and 300 U per mL recombinant *N*-glycosidase F cloned from *Flavobacterium meningosepticum* (Roche), the sample was incubated at 37°C overnight. Bovine fetuin (Sigma) and human transferrin (Roche) were used as positive controls. In contrast to N-linked carbohydrates, O-linked oligosaccharides may be difficult to remove from a protein in one step. O-deglycosylation studies were therefore performed by sequential treatment of dermal extracts first with neuraminidase from *Arthrobacter ureafaciens* (400 mU/mL) and then *O*-glycosidase from *Diplococcus pneumoniae* (200 mU/mL) at 37°C for 12 h, respectively (both enzymes from Roche). Deglycosylation of bovine fetuin (Sigma), known to contain neuraminic acid and O-linked sugars, was used as a positive control and monitored by Coomassie staining. Finally, to assess the presence of chrondroitin sulfate and heparan sulfate side chains in the p200 molecule, dermal extracts were digested with 4 U per mL chondroitinase ABC from *Proteus vulgaris* (in 0.1 M Tris-HCl, pH 8.0, containing 30 mM sodium acetate) or 100 mU per mL heparitinase I from *Flavobacterium heparinum* (in 20 mM sodium acetate, pH 7.0, containing 1 mM calcium acetate) at 37°C overnight. Heparitinase was from Seikagaku (Tokyo, Japan) and chondroitinase from Calbiochem (Schwalbach, Germany). Heparan sulfate from bovine kidney (Sigma) was used as a positive control for heparitinase digestion and the reaction was monitored at 232 nm. Negative controls for all deglycosylation experiments were incubated under identical conditions without addition of glycosidases. All enzymes used were of protease-free quality.

Two-dimensional gel electrophoresis was performed as described by O'Farrel (1975) with some modification. Dermal extracts were exhaustively dialyzed against double-distilled water and precipitated protein pelleted by centrifugation at $100{,}000 \times g$ for 1 h. The pellet was resuspended in 1/10 original volume of 2D sample buffer (9 M urea, 4% Triton X-100, 10% β-mercaptoethanol, 2% ampholines 3.5–10 (Sigma)) and separated by isoelectric focusing (first dimension) using 125-mm 3% polyacrylamide tube gels over 5000 Vh. After equilibration of the focused gels in Laemmli sample buffer, they were loaded on 6% polyacrylamide gels and subjected to SDS-PAGE (second dimension) in a xi electrophoresis cell (Bio-Rad). The two-dimensionally separated dermal proteins were then electrophoretically transferred to nitrocellulose membrane and probed with p200-specific antibodies.

N-terminal sequencing and mass spectrometry For N-terminal sequencing, two-dimensionally separated dermal extracts were transferred to polyvinylidenedifluoride membrane and the immunoreactive spot was analyzed using an ABI 492 CLC sequencing system (Applied Biosystems, Foster City, CA) following the instructions of the manufacturer. Mass spectrometry analysis was performed as described by Butt *et al* (2000). Briefly, the protein spot was excised from the gel and transferred to a quartz vessel. Gel pieces were dehydrated under vacuum and soaked with trypsin solution (0.05 μg/μL trypsin; Promega, Madison, WI). After digestion, the peptides were extracted with 8 μL of 5% formic acid for 15 min in the sonication bath and analyzed using an ESI-ion trap mass spectrometer (LCQ Classic, Thermo Finnigan, San Jose, CA) directly coupled to a nano-HPLC system (LC-Packings, Dionex, Idstein, Germany). For fully automated interpretation of the mass spectra, the NCBI protein database and the EST database were searched using the SEQUEST algorithm.

RESULTS

Anti-p200 autoantibodies belong exclusively to the IgG4 subclass Sera from six patients with anti-p200 pemphigoid were assessed for IgG subclass distribution of p200-specific autoantibodies. Representative results of this analysis are shown

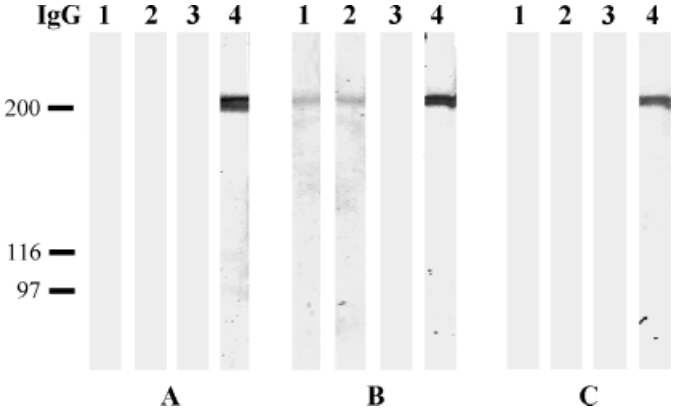

Figure 1. The autoimmune response in anti-p200 pemphigoid is mediated by IgG4 antibodies. Dermal extracts were separated by SDS-PAGE and studied by immunoblotting using serum from three patients with anti-p200 pemphigoid (*A–C*, respectively) and secondary monoclonal antibodies against human IgG1-IgG4 (*strips 1–4* of each panel, respectively). Molecular weight markers (kDa) are indicated on the left.

in **Fig 1**. In all six sera anti-p200 reactivity was mediated by IgG4 autoantibodies. Only one serum demonstrated weak additional IgG1 and IgG2 reactivity.

Efficient extraction of p200 occurs only after reduction of disulfide bonds and the extracted protein is insoluble in nonurea buffers As shown in **Fig 2(*A*)**, under nonreducing conditions, a buffer containing 8 M urea can solubilize only a trace amount of p200, whereas after incubation with 4 M urea, no extractable protein can be detected by immunoblotting. In contrast, reduction of disulfide bonds with β-mercaptoethanol enables significant amounts of p200 to be solubilized by both urea concentrations. Pretreatment of the dermis with bacterial collagenase fails to improve the extractability of p200 under nonreducing conditions. The solubility of p200 obtained by extraction with 8 M urea plus β-mercaptoethanol was determined in decreasing concentrations of urea, a urea-free buffer containing a mixture of three detergents (RIPA), and a high-salt buffer without urea or detergents (TBS). The results of this analysis are presented in **Fig 2(*B*)**. Reduction of the urea concentration by 50% caused roughly 50% of p200 to precipitate. Approximately 60% of originally extracted protein remained soluble in 4 M, 20% in 2 M, and 10% in 1 M urea. p200 was completely insoluble in nonurea buffers. Addition of detergents did not improve the solubility of the protein.

p200 is not produced by human keratinocytes or fibroblasts in mono- or coculture Total cellular extracts and concentrated conditioned medium of both cultured human keratinocytes and fibroblasts were separated by SDS-PAGE and studied by immunoblotting. The results of this analysis are presented in **Fig 2(*C*)**. Although cultured cells were demonstrated to produce various basement membrane proteins, including BP180, the unprocessed (200-kDa) and processed (165-kDa) forms of the α3 chain of laminin 5, and type VII collagen, sera from patients with anti-p200 pemphigoid did not react with any protein derived from the cellular extract or medium. To investigate the possibility that p200 requires the presence of both keratinocytes and fibroblasts for its expression *in vitro*, organotypic cocultures of human keratinocytes and fibroblasts were studied by indirect immunofluorescence microscopy using p200-specific sera. While type VII collagen was revealed as continuous lining at the epithelial–mesenchymal junction of cocultures, no deposition of p200 was detected (**Fig 2*D***).

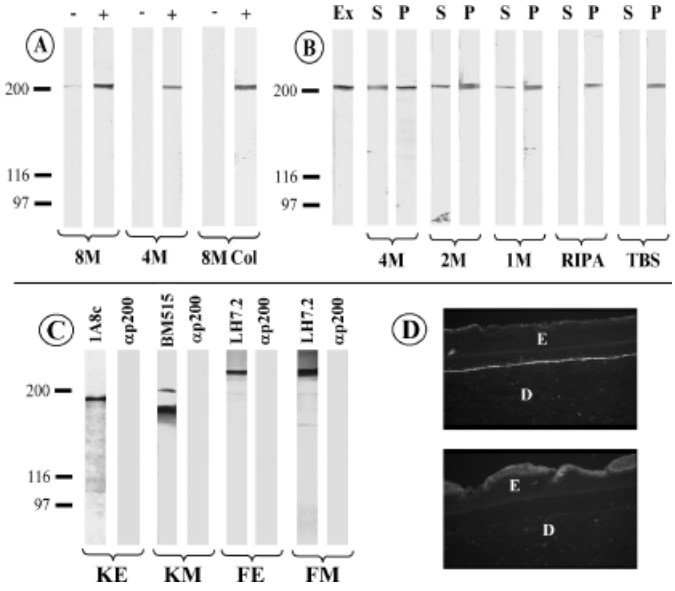

Figure 2. (*A*) Efficient extraction of p200 is reduction-dependent; (*B*) P200 is insoluble in nonurea buffers; (*C*) P200 is not produced by human keratinocytes or fibroblasts in culture; and (*D*) P200 is not expressed in organotypic coculture of human keratinocytes and fibroblasts. (*A*) Human dermis was extracted with 8 M urea (8 *M*), 4 M urea (4 *M*), or 8 M urea preceded by collagenase digestion (8 *M Col*) with (+) or without (–) addition of β-mercaptoethanol. Extracts were separated by SDS-PAGE and studied by immunoblotting using serum samples from patients with anti-p200 pemphigoid. Molecular weight markers are indicated on the left. (*B*) Dermal extracts, obtained by extraction with 8 M urea under reducing conditions (*Ex*) were dialyzed against 4, 2, or 1 M urea; RIPA buffer; or Tris-buffered saline (*TBS*) and centrifuged at 100,000 × *g*; supernatants (*S*) and pellets (*P*) were assessed for the presence of p200 by immunoblotting. (*C*) Monoclonal antibody 1A8c recognizes BP180 in total cellular extracts of human keratinocytes (*KE*) whereas monoclonal antibody BM515 stains both the unprocessed (200 kDa) and the processed (165 kDa) α3 chain of laminin 5 in concentrated conditioned medium (*KM*) from HaCaT cells. Monoclonal antibody LH7.2 detects type VII collagen in both total cellular extracts (*FE*) and medium (*FM*) of cultured human fibroblasts. P200-specific sera samples (*αp200*) are unreactive with these substrates. (*D*) Whereas the monoclonal antibody LH7.2 demonstrates continuous deposition of type VII collagen along the epithelial–mesenchymal junction (*top*), no expression of the p200 antigen is detected after 3 wk of coculture (*bottom*). *E*, epidermis; *D*, dermis.

P200 is a noncollagenous protein of the DEJ To determine whether p200 is a member of the collagen family, dermal extracts were digested with bacterial collagenase and assayed for reactivity with sera from patients with anti-p200 pemphigoid and monoclonal antibody LH7.2 against human type VII collagen. Even prolonged incubation with bacterial collagenase did not result in degradation of p200 (**Fig 3**). In contrast, full-length type VII collagen (290 kDa) was digested to its collagenase-resistant NC1 domain (145 kDa) within 1 h of collagenase treatment. With longer incubation times, the 145-kDa band increased in sharpness but was not degraded any further, confirming the specificity of collagenolytic activity. Similar results were obtained using dermal type I and type VI collagens visualized by immunoblotting with specific rabbit antisera as controls (not shown). When dermal extracts were incubated at 37°C without collagenase, p200 and type VII collagen remained intact, as did bovine serum albumin subjected to collagenase digestion. Treatment of dermal extracts with bacterial protease V8 resulted in complete loss of p200 reactivity within 30 min after addition of the protease. No immunoreactive fragments could be detected even after

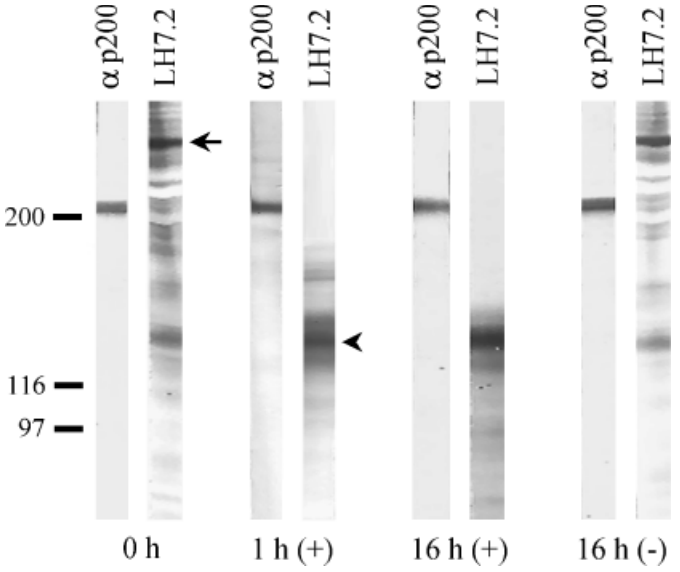

Figure 3. p200 is a noncollagenous protein. Dermal extract was digested with bacterial collagenase as described in the text. Extract before digestion (*0 h*), after digestion [(*1 h (+), 16 h (+)*], and control extract incubated without collagenase at 37°C for 16 h (*16 h (–)*) were assayed for immunoreactivity with serum from patients with anti-p200 pemphigoid (*αp200*) and monoclonal antibody against type VII collagen (*LH7.2*). *Arrow*, full-length type VII collagen (290 kDa); *arrowhead*, NC1 domain of type VII collagen (145 kDa).

separation of digestion products in a 4% to 20% gradient gel (not shown).

p200 is an N-linked glycoprotein *N*-Glycosidase F (PNGase) is capable of selectively removing all classes of asparagine-bound glycans from the protein backbone (Tarentino and Plummer, 1987). Incubation of dermal extracts with *N*-glycosidase F resulted in an increase of electrophoretic mobility of the p200 autoantigen (**Fig 4**, *left*). The deglycosylated protein migrated almost exactly midway between the intact p200 in dermal extracts and BP180 recognized by rabbit serum SA8010 in

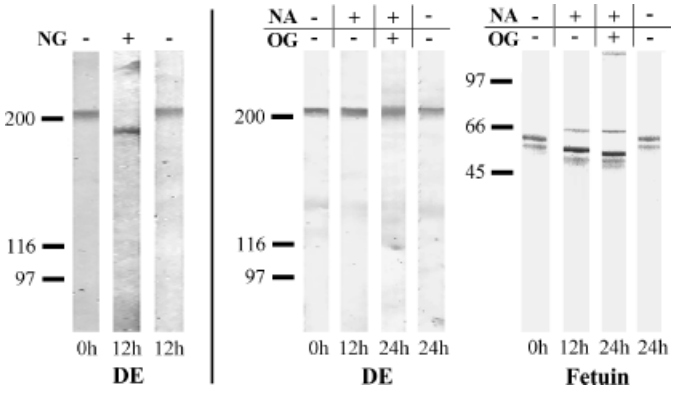

Figure 4. p200 is an N-linked glycoprotein. (*Left*) *N*-glycosylation studies: Dermal extract (*DE*) was digested with *N*-glycosidase F (*NG*). Extract before digestion (*0 h*), after digestion (*12 h; +*), and control extract incubated without glycosidase (*12 h; –*) were separated by SDS-PAGE and analyzed for immunoreactivity with serum from patients with anti-p200 pemphigoid. (*Right*) Sialylation and O-glycosylation studies: Dermal extract (*DE*) and bovine fetuin were digested first with neuraminidase (*NA*) and then with *O*-glycosidase (*OG*). The p200 autoantigen was visualized by immunoblotting and fetuin by Coomassie blue staining. +, with enzyme; –, without enzyme. Incubation periods are indicated below the respective strips.

extracts of cultured human keratinocytes (not shown). Therefore, the extent of N-glycosylation of p200 was estimated at approximately 10 kDa. Negative controls incubated without enzyme did not show any nonspecific degradation of p200, whereas digestion of bovine fetuin and human transferrin resulted in reduction of their apparent molecular weights to the expected sizes of N-deglycosylated proteins.

p200 is not O-glycosylated and is devoid of chondroitin sulfate and heparan sulfate side chains The most common O-linked structure found in glycoproteins is Gal-β-1,3 GalNAc that can be successfully removed by *O*-glycosidase (Endo and Kobata, 1976). Nevertheless, modification of this core structure, usually by sialylation, can block the action of the enzyme and needs to be removed by a suitable neuraminidase (sialidase). Therefore, to investigate the presence of O-linked oligosaccharides on the polypeptide backbone of p200, dermal extracts were sequentially treated first with neuraminidase and then with *O*-glycosidase. This treatment did not result in a reduction of apparent molecular weight of the p200 molecule, whereas bovine fetuin showed an expected increase in electrophoretic mobility after each deglycosylation step (**Fig 4**, *right*). Hence, sialic acid and O-glycans appear to be absent in p200. For further characterization of the glycosylation status of p200, dermal extracts were treated with bacterial chondroitinase ABC and heparitinase I. After digestion with chondroitinase, no shift in electrophoretic mobility of p200 was observed. In contrast, proteoglycan I (biglycan), also present in dermal extract, was efficiently degraded to its 47-kDa core protein as detected by immunoblotting with a biglycan-specific rabbit antibody. Similarly, heparitinase I had no effect on the apparent molecular weight of the p200 molecule, while it rapidly hydrolyzed bovine heparan sulfate as evidenced by an increased adsorption at 232 nm (not shown).

All p200 sera target an identical dermal protein To demonstrate that all six anti-p200 sera bind the same molecule, we electrophoresed extracts of normal human dermis in two dimensions (isoelectric focusing followed by SDS-PAGE). Separated proteins were transferred to nitrocellulose for immunoperoxidase staining. **Figure 5** shows that different p200 sera identify the same major acidic spot at pI 5.4 to 5.6 and molecular weight of 200 kDa. The spots recognized by different patient sera comigrate with each other and with the 200-kDa protein stained by the same sera in dermal extracts separated only by SDS-PAGE. Normal human sera did not label this spot.

N-terminal sequencing and mass spectrometry analysis of the immunoreactive spot In an attempt to determine the sequence of the p200 autoantigen, the immunoreactive spot of two-dimensionally separated dermal extracts was subjected to N-terminal sequencing. However, the protein concentration of the spot proved to be too low for this analysis. Alternatively, the immunoreactive spot was studied by mass spectrometry directly coupled to a nano-HPLC system. Mass spectrometry repeatedly identified the α3 chain of type VI collagen and filamin A as the major components of the spot. No other proteins could be detected even when using 10-fold concentrated dermal extract and narrow range high-resolution ampholines for isoelectric focusing. To determine whether p200 is identical to type VI collagen or filamin A, purified human type VI collagen (Rockland) and recombinant full-length human filamin A (gift of J.H. Hartwig, Boston, MA) were studied by immunoblotting using serum from patients with anti-p200 pemphigoid. However, all patients' sera were unreactive with both proteins (not shown).

DISCUSSION

The cutaneous basement membrane is a complex network of interconnecting proteins, including the extracellular domains of

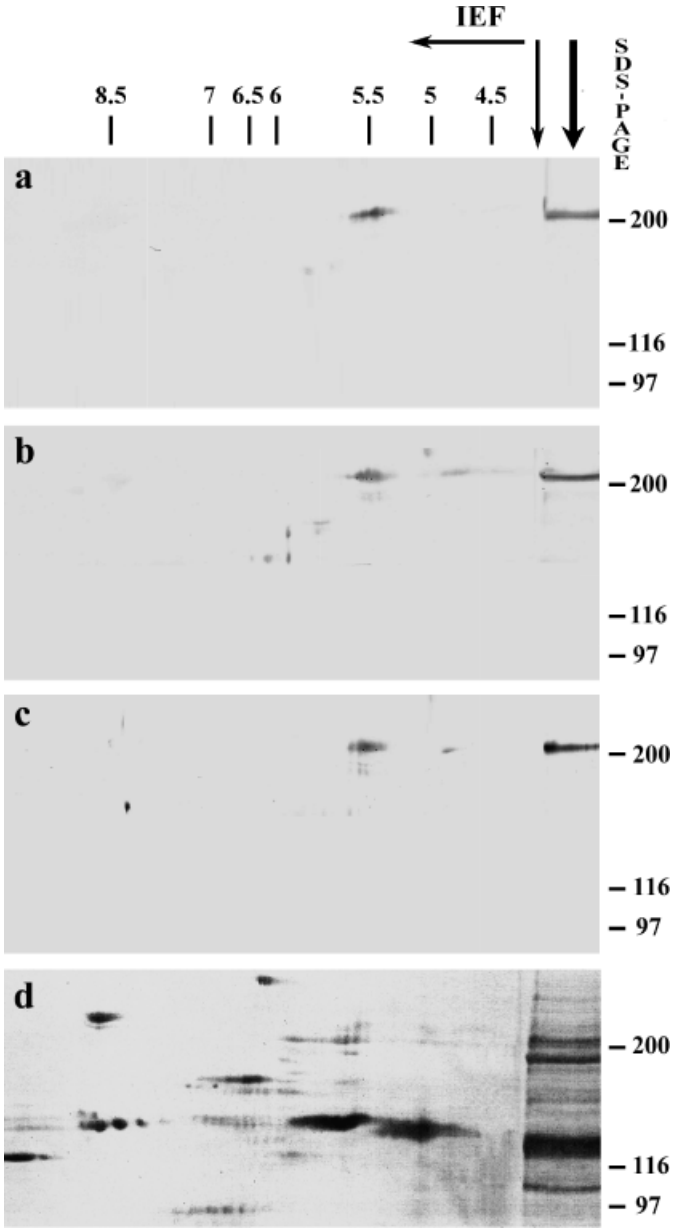

Figure 5. All patients with anti-p200 pemphigoid recognize an identical protein of two-dimensionally separated dermal extracts. Proteins in extracts of normal human dermis were separated by isoelectric focusing (*IEF*) in the horizontal dimension and then by SDS-PAGE in the vertical dimension (*thin downward arrow*). Control extracts were separated in the same gels only by SDS-PAGE (*thick downward arrow*). Proteins were transferred to nitrocellulose sheets and either probed by sera from three different patients with anti-p200 pemphigoid (*a–c*) or stained with Fast Green (*d*). Note that immunoreactive spots in *a–c* comigrate with each other at pI 5.4 to 5.6 and with the 200-kDa immunoreactive bands of control extracts. Migration positions of pI markers are indicated along the upper margin, and those of molecular weight markers along the right margin.

BP180 and α6β4 integrin; laminins 1, 5, and 6; nidogen; type IV and VII collagen; fibronectin; and perlecan (Burgeson and Christiano, 1997). Interestingly, none of these proteins appears to be identical to the p200 autoantigen. In contrast to anti-p200 antibodies, those against BP180 and α6β4 integrin stain the epidermal side of salt-split skin (Tyagi *et al*, 1996; Sitaru *et al*, 2002). p200-specific sera do not react with purified laminins 1, 5, and 6 (Zillikens *et al*, 1996; Kawahara *et al*, 2000) and show linear staining of the basement membrane by indirect immunofluorescence microscopy of laminin 5-deficient human skin (Zillikens *et al*, 2000). In addition, by immunoblotting, monoclonal antibodies BM515 and A1 against the α3 chain of laminin 5 and the γ1 chain of laminin 1, respectively, do not label the same protein in dermal extracts compared to p200-specific autoantibodies. Nidogen can be excluded as a candidate owing to its molecular weight of 80 kDa (Timpl *et al*, 1983). In this study, we show that p200 is resistant to digestion with collagenase, heparitinase, and chondroitinase, thus eliminating type IV and VII collagen, perlecan, and other proteoglycans as possible targets of p200-specific autoantibodies. Moreover, monoclonal antibody LH7.2 against type VII collagen does not recognize a 200-kDa protein in dermal extracts. Finally, we determined that anti-p200 sera do not react with purified human fibronectin. Taken together, these data suggest that p200 may represent a hitherto unknown component of the cutaneous basement membrane.

IgG4 was demonstrated to be the dominant autoantibody subclass in many autoimmune blistering skin diseases, including pemphigus, BP, mucous membrane pemphigoid, and epidermolysis bullosa acquisita (Yamada *et al*, 1989; Bernard *et al*, 1990, 1991; Döpp *et al*, 2000). However, in most patients with these diseases, the predominance of IgG4 is not absolute and other subclasses are also encountered in the majority of cases. Interestingly, in patients with anti-p200 pemphigoid, p200-specific reactivity was mediated exclusively by IgG4. Such stringent isotype restriction presents an important advantage for any studies on the p200 autoantigen, as a monoclonal IgG4-specific secondary antibody can be used for its immunodetection.

In the past, differential extraction experiments have been successfully employed in defining components and organization of the ECM. In our study, a two-step extraction protocol was used. In the first step, human dermis was extracted with 8 M urea without addition of a reducing agent. Such treatment is known to solubilize proteins noncovalently associated with the ECM (Carter, 1982). In the second step, ECM components stabilized by intermolecular disulfide bonds were extracted with 8 M urea plus mercaptoethanol. The p200 autoantigen resisted extraction by 8 M urea alone but was readily released from the dermis once disulfide bonds were disrupted. These findings suggest that p200 molecules form highly insoluble S-S-dependent complexes. These complexes may represent homomultimers of p200, similar to those described for type VII collagen (Bentz *et al*, 1983; Morris *et al*, 1986). Alternatively, p200 may be anchored to (an)other poorly soluble component(s) of the ECM. Examples of such covalent protein–protein interactions have been described between desmogleins and plakoglobin (Eyre and Stanley, 1988) as well as between laminins 5 and 6/7 (Champliaud *et al*, 1996). Collagenase digestion of the dermis did not increase the yield of p200 under nonreducing conditions. Assuming that p200 is indeed a part of a heterocomplex stabilized by disulfide bonding, this observation suggests that collagenous proteins are not involved in its formation.

To determine whether p200 can be maintained in solution under milder conditions than those required for its initial extraction, dermal extracts were dialyzed against decreasing concentrations of urea, a combination of detergents, and a high-salt buffer without urea or detergents. Minor amounts of p200 remained soluble in the presence of low urea concentrations, whereas removal of urea resulted in complete precipitation of the protein. These results should be important for the further characterization of p200 because they virtually preclude the use of first choice techniques such as immunoprecipitation or immunoaffinity chromatography for its isolation.

To determine the cellular origin of the p200 autoantigen and to identify a source of the protein soluble in nonurea buffers, we examined total cellular extracts and concentrated conditioned medium of both human keratinocytes and fibroblasts for the presence of p200. In addition, we studied the reactivity of sera from patients with anti-p200 pemphigoid with organotypic cocultures of keratinocytes and fibroblasts. These cocultures have previously

been demonstrated to deposit a well-organized basement membrane *in vitro*, containing laminins 1 and 5, nidogen, and type IV and type VII collagen (Smola *et al*, 1998). Unfortunately, p200-specific sera did not react with these substrates. It is therefore likely that conditions required for deposition of p200 *in vivo* are not correctly reproduced in mono- and coculture of cutaneous cells.

Various collagen types have been implicated in human autoimmune blistering diseases. Type VII collagen is a well-characterized autoantigen of epidermolysis bullosa acquisita, type XVII collagen/BP180 is the common autoantigen in different pemphigoid diseases, and the α5 chain of type IV collagen has been recently described as the antigenic target in patients with a novel autoimmune subepidermal blistering disease (Woodley *et al*, 1984; Ghohestani *et al*, 2000; Zillikens, 2002). Interestingly, we found p200 to be highly resistant to digestion with bacterial collagenase, whereas major dermal collagens (types I, VI, and VII) were efficiently degraded by the enzyme. This result strongly suggests that p200 is a noncollagenous protein and confirms our previous data demonstrating that p200 is distinct from type VII collagen (Zillikens *et al*, 2000).

We demonstrate that p200 is a glycoprotein carrying approximately 10 kDa of N-linked carbohydrates on its polypeptide backbone. Such extent of glycosylation would indicate a presence of about three N-linked oligosaccharide branches within the p200 molecule. Neuraminic acid residues and O-linked glycan chains appear to be absent from p200 because the glycoprotein is resistant to digestion with neuraminidase and *O*-glycosidase. Desmogleins 1 and 3, BP180, and type IV and type VII collagen as well as various laminin chains have all been demonstrated to possess some form of glycosylation (Sage *et al*, 1979; Stanley *et al*, 1982; Woodley *et al*, 1986; Penn *et al*, 1987; Knibbs *et al*, 1989; Nishizawa *et al*, 1993; Pas *et al*, 1997; Schumann *et al*, 2000). Although the biologic functions of carbohydrate moieties are not yet fully understood, N-glycans of laminin were shown to be important for cell adhesion and spreading as well as for neurite outgrowth formation (Dennis *et al*, 1984; Dean *et al*, 1990) while unglycosylated type VII collagen could not be secreted by cultured cells into the medium (Chen *et al*, 1997). In addition, carbohydrate antigenic determinants were demonstrated to be important for autoantibody response in several diseases, including pernicious anemia, chronic Chagas' disease, rheumatoid arthritis, and endometriosis (Goldkorn *et al*, 1989; Almeida *et al*, 1994; Blass *et al*, 1998; Lang and Yeaman, 2001). Removal of asparagine-linked oligosaccharides from p200 did not affect antigen recognition by patients' autoantibodies, indicating that its epitopes are glycosylation-independent. Interestingly, the same is true for antigenic determinants recognized by pemphigus autoantibodies on desmogleins 1 and 3 and BP-specific epitopes on BP180 (Amagai *et al*, 1995; Pas *et al*, 1997; Schumann *et al*, 2000).

Since its first description in 1996, 14 cases of anti-p200 pemphigoid have been published (reviewed in Watanabe *et al*, 2002). Although antibodies in sera of all these patients recognized a dermal protein of 200 kDa by immunoblotting, certain dissimilarities in clinical presentation, histopathologic findings, and response to treatment raised questions with regard to the common identity of the target molecule in these patients. In addition, some degree of heterogeneity in the apparent molecular weight of the 200-kDa antigen was reported (Mascaro *et al*, 2000). In this study, we demonstrate that as many as six patients with anti-p200 pemphigoid recognize an identical dermal protein as defined by molecular weight, isoelectric point, collagenase resistance, and extent of glycosylation. These findings strongly suggest that anti-p200 pemphigoid forms a single entity characterized by an autoimmune response against a unique component of the cutaneous basement membrane.

In an attempt to determine the amino acid sequence of the p200 autoantigen, the immunoreactive spot of two-dimensionally separated dermal extracts was studied by Edman degradation and mass spectrometry. Although the protein concentration of the spot proved to be too low for N-terminal sequencing, mass spectrometry identified the α3 chain of type VI collagen and filamin A. Type VI collagen is a ubiquitous collagenous heterotrimeric protein composed of α1 and α2 chains, each of 140 kDa, and an α3 chain of 200 kDa (Trüeb and Winterhalter, 1986). In the skin, type VI collagen is produced by dermal fibroblasts and deposited throughout the reticular and papillary dermis as detected by indirect immunofluorescence microscopy (Hessle and Engvall, 1984). Immunoelectron microscopic studies demonstrated that dermal type VI collagen forms a microfibrillar network closely associated with interstitial collagen but does not localize to the cutaneous basement membrane or anchoring plaques (Keene *et al*, 1988). Filamin A is a 250-kDa cytoskeletal protein of smooth muscle and nonmuscle cells (Wang *et al*, 1975). In the skin, filamin is expressed in the peripheral cytoplasm of keratinocytes and is not present in the ECM (Kubler and Watt, 1993). Thus, biochemical properties and ultrastructural localization of type VI collagen and filamin A are quite different from those of p200, a noncollagenous protein of the cutaneous basement membrane. In fact, sera from patients with anti-p200 pemphigoid did not react with purified type VI collagen or recombinant full-length filamin A. The finding that all p200-specific sera unequivocally recognize the same spot of two-dimensionally separated dermal extracts but remain unreactive with its identifiable components raises two possibilities: (1) autoantibodies from patients with anti-p200 pemphigoid may recognize unique epitope(s) not present within full-length type VI collagen or filamin A. In fact, most sera samples from patients with linear IgA disease react with cleavage-derived neoepitopes on the LABD97 and LAD-1 autoantigens but not with the full-length BP180 molecule (Zone *et al*, 1990; Pas *et al*, 1997; Schumann *et al*, 2000). (2) Another protein, distinct from both type VI collagen and filamin A, may be present within the immunoreactive spot. This protein may not be detected by mass spectrometry owing to its low concentration or owing to the absence of its sequence from the available databases. The experimental evidence presented above rather supports the latter possibility. Owing to the limits imposed by poor solubility of dermal proteins, we were unable to concentrate dermal extract by more than a factor of 10. Our attempts to purify p200 by a combination of collagenase digestion and ion-exchange chromatography resulted in too much loss of immunoreactive protein. The enrichment of dermal extract for p200 may therefore be insufficient to identify the protein using a purely biochemical approach. Our future efforts will be directed at screening a human skin cDNA library with p200-specific autoantibodies. After initial library screens, the new insights into the biochemistry of this autoantigen, provided by the present study, should be helpful for the selection of proteins warranting further investigation.

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